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Effect of Glutamine on Methotrexate Efficacy and Toxicity

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Objective

To examine the effect of oral glutamine (GLN) on the efficacy and toxicity of methotrexate (MTX).

Summary Background Data

The use of high-dose chemotherapy regimens is limited by the severity of their toxicities. Oral GLN has been shown to decrease the gut toxicity seen with MTX treatment while enhancing its tumoricidal effect.

Methods and Results

Studies were done in laboratory rats and in breast cancer outpatients. Fischer 344 rats were randomized to 48 hours of prefeeding with GLN (1 g/kg/day) or an isonitrogenous amount of glycine. Rats were killed 24 hours after receiving a 20-mg/kg intraperitoneal dose of MTX. In the GLN group, there was a threefold increase in total MTX in the tumor as compared with the control group, and this increase was in both the diglutamated and pentaglutamated MTX. Inversely, there was a significant decrease in the total polyglutamated MTX in the gut in the GLN group.

Given the results of this preclinical study, the authors performed a phase I trial. Nine patients diagnosed with inflammatory breast cancer received GLN (0.5 g/kg/day) during MTX neoadjuvant therapy, escalating from doses of 40 mg/m^2 to 100 mg/m^2 weekly for 3 weeks, followed by a doxorubicin-based regimen. No toxicity of oral GLN was detected. No patient showed any sign of chemotherapy-related toxicity. One patient had a grade I mucositis. Except for one, all patients responded to the chemotherapy regimen. Median survival was 35 months.

Conclusions

These studies suggest that GLN supplementation is safe in its administration to the tumor-bearing host receiving MTX. By preferentially increasing tumor retention of MTX over that of normal host tissue, GLN may serve to increase the therapeutic window of this chemotherapeutic age.

Catabolic states such as major surgery, sepsis, and cancer are characterized by alterations in the interorgan exchange of amino acids, net skeletal muscle breakdown, and negative nitrogen balance.[1] Toxicity to the tumor-bearing host is seen from the disease and from chemotherapy-related injury. Dose intensification of chemotherapy is thought to increase survival.[2,3] However, the limitation of intensifying chemotherapeutic regimens has become the severity of toxicity to the normal host tissues.[4]

Glutamine (GLN) is a nonessential amino acid that serves not only as a primary respiratory fuel but also as a necessary substrate for nucleotide synthesis in most dividing cells.[5] GLN is required by enterocytes, lymphocytes, and fibroblasts as well as by rapidly growing tumors.[6–8] Fox et al.[9,10] described the benefits of a GLN-enriched diet in rats receiving methotrexate (MTX) chemotherapy. The GLN-enriched diet improved nutritional status, decreased intestinal injury, decreased bacterial translocation, and resulted in an improved survival in a lethal model of enterocolitis. Other studies from the authors' laboratory demonstrated that the provision of dietary GLN to the cancer-bearing host nearly doubled the tumoricidal action of MTX while reducing its morbidity and mortality in the host.[11,12] Animals had less enterocolitis, improved hematologic parameters, decreased sepsis, and improved survival in a rat sarcoma model receiving multiple doses of MTX. Using a single high-dose MTX rat model, the authors demonstrated increased retention of MTX in tumor cells.[13]

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The authors hypothesized that GLN's ability to increase MTX concentrations in tumor cells is by increasing the amount of polyglutamated MTX, which is preferentially retained by the tumor. To study this, the authors first examined concentrations of polyglutamated MTX in tumor as well as gut from animals receiving high-dose MTX. They then conducted a phase I trial of supplemental oral GLN with escalating doses of MTX in patients with inflammatory breast cancer.

MATERIALS AND METHODS

Animal Studies

Male Fischer 344 rats weighing 300 g were obtained from SASCO (Omaha, NE). All studies were approved by the Animal Care and Use Committee at the John L. McClellan Memorial Veterans Administration Hospital, Little Rock, AR. The rats were maintained in cages in the animal care facility, subjected to alternate 12-hour periods of dark and light, and were given at least 1 week to acclimate to the animal care facility. Eighteen rats received flank implantation of $2 \times 2 \times 2$ mm^3 of viable 3-methylcholanthrene-induced fibrosarcoma cells after anesthesia was obtained with ketamine hydrochloride (7.5 mg/100 g) and acepromazine maleate (0.1 mg/100 g).

At day 23 after implantation, rats were randomized to receive 1 g/kg/day of GLN (n = 9) or an isonitrogenous amount of glycine (GLY, n = 9) by gavage. Rats were allowed *ad libitum* intake of chow and water and were pair-fed. On day 25 after implantation, all rats received a single intraperitoneal dose of 20 mg/kg MTX. Rats were killed 24 hours after initiation of MTX (day 26).

All rats were weighed and anesthetized using ketamine hydrochloride (7.5 mg/100 g) and acepromazine maleate (0.1 mg/100 g). Under sterile conditions, a midline incision was made and the rat heparinized. Arterial blood was taken from the aorta using a 25-gauge needle attached to a 1-mL syringe. Serum was processed for arterial GLN and MTX concentrations. A 10-cm section of jejunum was processed for glutaminase and MTX concentrations. The tumors were measured, weighed, and assayed for glutaminase activity and MTX concentration and processed for morphometrics.

Aliquots of heparinized whole blood were mixed with equal volumes of cold 10% perchloric acid, then vortexed and centrifuged at 5° C at 3000*g* for 10 minutes. The supernatant was removed and neutralized with an equal amount of cold 0.48 M potassium phosphate. This was again vortexed and centrifuged at 5° C at 3000*g* for 10 minutes. The supernatant was removed and kept frozen at −20° C to later determine the GLN concentration by the microanalytic method described by Bergmeyer[14] and to measure the MTX concentration.

After removing the tumors from the flank, a 0.5-g portion of the tumor was homogenized with 50 mmol of sodium phosphate for 1 minute. An aliquot of this mixture was taken to measure protein concentration,[15] glutaminase activity,[16] and MTX.[17,18] MTX assays were preformed after freezing and centrifugation using high-pressure liquid chromatography (HPLC), as described in detail below. Material for light microscopy was immediately fixed in 10% buffered formaldehyde solution and submitted dehydrated and embedded in historesin plastic media. Several tissue sections of 6 μm were cut from each plastic block and mounted on glass slides. The slides were stained with hematoxylin and eosin.

Mucosa from a 10-cm section of proximal jejunum was scraped separately and homogenized in 50 mmol of sodium phosphate buffer containing 300 mmol of sucrose. An aliquot of this mixture was then removed for glutaminase activity and protein determination. Protein concentration was determined by the method of Lowry et al.[15] Phosphate-dependent glutaminase activity was determined using a microfluorometric assay similar to that described by Windmueller.[16]

For HPLC, the tumor was weighed, minced, and dropped into the prehot buffer (2% sodium ascorbate, 10 mM 2-mercaptoethanol in 0.1 M Bis-Tris, pH 7.85). It was boiled for 20 minutes. The extract was cooled in an ice-water bath and dispersed by homogenization, and the volume was adjusted to make a final suspension that corresponded to 10 volumes per gram wet tissue. This extract was then centrifuged at 36,000*g* for 15 minutes, and the supernatant fraction was injected into a vacutainer and stored at −70° C.[17] On analysis, the extract was added to ice-cold 10% trichloroacetic acid in a 1:4 ratio, then centrifuged at 3000*g* for 15 minutes; the supernatant was saved. A BAKERBOND spe C_{18} column (J. T. Baker, Phillipsburg, NJ) was prepared by rinsing 50% acetonitrile (ACN) followed by water. The supernatant was applied to the column and was washed with water. The MTX polyglutamates were then eluted with 50% ACN. The sample was evaporated to dryness under vacuum with centrifugation and was then resuspended in the HPLC mobile phase buffer.[18]

The MTX assay was performed using a model 600E pump, U6K injector, and 996 photodiode array detector (Waters, Milford, MA). Sample aliquots were injected on a 3.9×150 mm C_{18} μBondapak column (Waters) that had been equilibrated with 15% ACN in 5 mM tetrabutyl ammonium phosphate (PicA). The MTX polyglutamates were eluted at 1 mL/min along a gradient of 15% to 55% ACN/5 mM PicA for 20 minutes, followed by 55% ACN/5 mM PicA for the final 15 minutes of the separation.[18] The MTX polyglutamates were identified on the basis of retention time determined with authentic standards and using a standard method.

Human Studies

Patients 18 years and older with inflammatory breast cancer treated at the Arkansas Cancer Research Center and the University of Arkansas for Medical Sciences were eli-

gible for participation in this phase I trial. The study protocol was approved by the Institutional Review Board at the University of Arkansas for Medical Sciences. All the patients were diagnosed as having inflammatory breast cancer before the start of treatment by incisional biopsy or core biopsy (tru-cut, or stereotactic-guided). Informed consent was obtained from all the patients before starting treatment. Patients with uncontrolled, clinically significant lung, heart, endocrine, or renal disease, those who were nursing or pregnant, or those who had any other cancer were excluded from the trial. Patients were also excluded if they had any known hypersensitivity to glutamate.

Nine patients were entered into this phase I trial. All patients received oral GLN supplementation (0.5 g/kg/day) starting 4 days before the start of chemotherapy, continuing during and for 1 week after treatment with MTX. Patients were entered into the trial in groups of two or three at the beginning dose of 40 mg/m^2 MTX. MTX was given weekly for a total of 3 weeks. This was followed by three monthly doses of 5-fluorouracil, doxorubicin, and cyclophosphamide, mastectomy (modified radical or simple extended), two monthly doses of the chemotherapy regimen after surgery, and radiation therapy (XRT) to the chest wall. Patients were to receive granulocyte colony-stimulating factor (5 mg/kg) on days 2 to 5 every week of chemotherapy for a low white blood count that would delay treatment. If no grade III or IV toxicities were seen, three additional patients were entered with MTX dosed at 60 mg/m^2, followed by 80 mg/m^2 and then 100 mg/m^2, otherwise following the same protocol.

Tumor response was evaluated comparing the clinical and pathologic size of tumor and the lymph node status. Toxicity evaluation was performed in accordance with the guidelines of the Southwest Oncology Group Toxicity Criteria (hematologic, infection, circulatory, clotting, cardiac, liver, lung, renal/bladder, gastrointestinal, neurologic, neuromotor, neurosensory, pain, dermatologic, immunologic, mucosal, metabolic, endocrine, and miscellaneous). These criteria are graded from 0 to 5; the toxicity grade should reflect the most severe degree occurring during the evaluated period. Hematologic toxicity referred to neutropenia (<2,500/μL) or a platelet deficiency (<75,000/μL) sufficient to cause a delay in the treatment. Liver and kidney toxicities were evaluated at baseline and weeks 2, 3, 4, 5, and 6 by analyzing transaminases (SGOT, SGPT, SGGT), bilirubin, alkaline phosphatase, urine urea nitrogen, creatinine, and blood urea nitrogen. MTX delivery would be delayed for a grade 3 elevation of the transaminases (more than five times normal) with a normal bilirubin level, or for a bilirubin >3 or for levels of creatinine 3.1 to 6 times normal. Mucositis during treatment with MTX was measured in accordance with the World Health Organization Toxicity Grading Scale (from grade 1 [pink and moist] to grade 3 [ulcerations]) at weeks 2, 3, and 4. Infection during chemotherapy treatment was diagnosed by clinical signs, cultures for aerobic and anaerobic organisms, or the need for oral or intravenous antibiotics at weeks 2, 3, and 4.

MTX was held for grade 4 nausea and vomiting (dehydration or electrolyte imbalance) that did not respond to antinausea medication. Development of local, regional, or distant progression of disease was grounds from removal from the study.

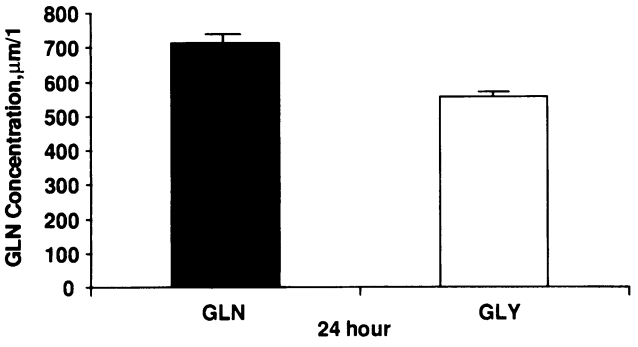

Figure 1. Arterial glutamine (GLN) concentration. Rats receiving supplemental GLN had elevated arterial GLN concentrations over those receiving the control diet at 24 hours. All values are mean ± SEM, $p < 0.05$.

Statistical Analysis

All data are expressed as mean ± standard error of the mean. Differences between means were considered significant at $p < 0.05$ using the unpaired two-tailed Student's t test (Macintosh IIci computer, Apple Computers, Cupertino, CA, and StatView II, Abacus Concepts, Berkeley, CA).

RESULTS

Animal Studies

Food intake was identical in both groups throughout the study period. There were no differences between body weights of rats in the GLN and GLY regimens within the two groups on the day of initiation of the study or at death. Body weights (321 ± 5 g in the GLN group *vs.* 311 ± 5 g in the GLY group, p = NS), chow intake (32.4 ± 1.3 g/day in the GLN group *vs.* 31.9 ± 0.9 g/day in the GLY group, p = NS), or gavage intake (14.3 ± 0.2 mL/day in the GLN group *vs.* 14 ± 0.5 mL/day in the GLY group, p = NS) were not significantly different between groups.

Rats receiving supplemental GLN had elevated arterial GLN concentrations over those receiving the control diet at 24 hours (Fig. 1).

On day 25, before injection of MTX or saline control, tumor volume was similar for the GLN and GLY regimens within the two groups. Initial tumor volume was 12.2 ± 2.9 cm^3 in the GLN group *versus* 11.7 ± 2.3 cm^3 in the GLY group (p = NS). Tumor volume loss after MTX was −2.4 ± 1.3 cm^3 in the GLN group *versus* −0.1 ± 0.9 cm^3

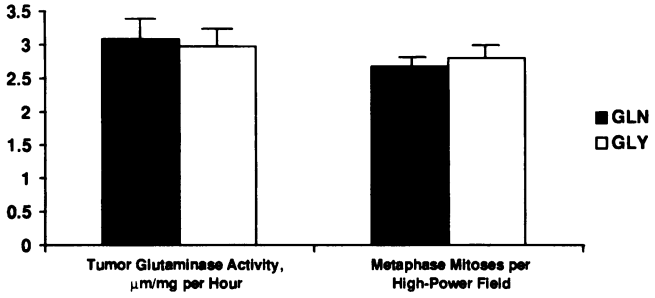

Figure 2. Effects of a glutamine (GLN) diet on tumor growth parameters. Tumor growth parameters were not significantly different between the GLN group and the glycine group.

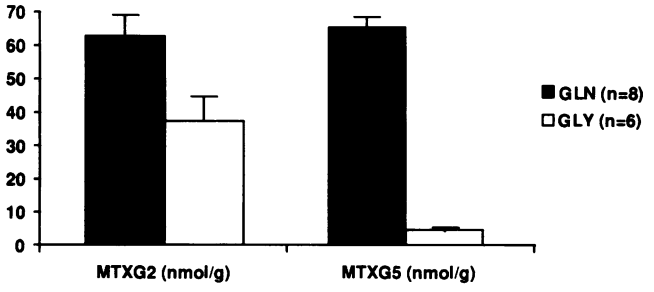

Figure 4. Diglutamated and pentaglutamated Methotrexate (MTX) in the tumor. The increase in MTX in the tumor occurred in both diglutamated and pentaglutamated MTX in the glutamine group compared with the control group.

in the GLY group ($p < 0.5$). Specific activity of glutaminase and tumor mitoses per high-power field were not different between groups (Fig. 2).

The provision of dietary GLN in rats receiving MTX increased MTX concentrations in tumor tissue at 24 hours by threefold over the control group (Fig. 3A). However, serum levels of MTX were not significantly different (Fig. 3B). This increase in total MTX in the tumor was a result of an absolute increase (nmol/g) in both the diglutamated and pentaglutamated MTX. There was a 14-fold increase of intracellular tumor pentaglutamated MTX in the GLN group over that seen in the GLY group (Fig. 4). Inversely, there was a significant decrease in total polyglutamated MTX in the gut of the GLN-fed animals *versus* the GLY-fed animals (see Fig. 3A). Specifically, in the gut there was a significant decrease in MTX6 (74.4 ± 7.4 nmol in the GLY group *vs.* 45.9 ± 8.33 nmol/g in the GLN group, $p < 0.05$).

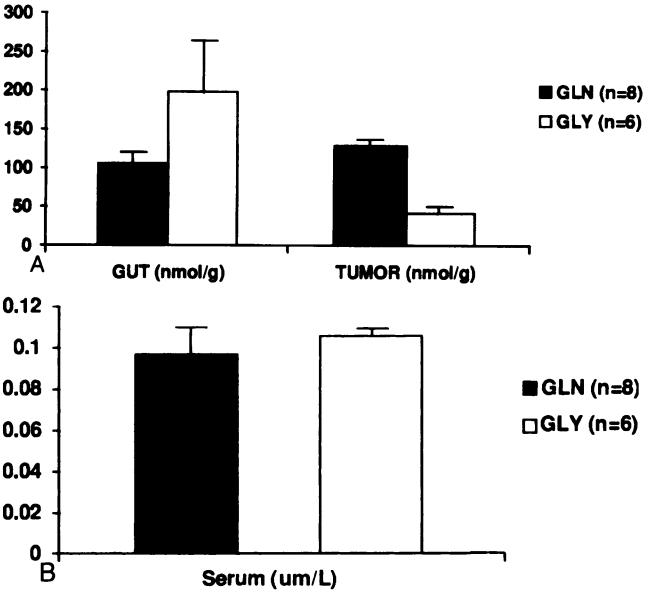

Figure 3. (A) Methotrexate (MTX) metabolism in the gut and tumor. In the glutamine (GLN) group, there was a threefold increase in total MTX in the tumor compared with the control group. Inversely, there was a decrease in total polyglutamated MTX in the gut in the GLN group. (B) MTX metabolism in the serum. There were no differences in the concentration of MTX in the serum between the GLN group and the control group.

Human Studies

Of the nine patients who entered the phase I trial, two were enrolled on the weekly dose level of 40 mg/m^2 MTX for the 3 weeks, three patients on 60 mg/m^2, two on 80 mg/m^2, and two on 100 mg/m^2 MTX for 3 weeks. Patient characteristics are shown in Table 1. The median age at diagnosis was 47 years (range, 39 to 72). The median follow-up time of living patients was 47 months (range, 29 to 57). One patient was lost to follow-up immediately after the completion of treatment.

No toxicity of oral GLN itself was detected in any patient. Chemotherapy was not held or delayed in any patient. One patient receiving 80 mg/m^2 of MTX had grade I mucositis, but no mucositis occurred in the patients receiving 100 mg/m^2. No patient had chemotherapy-related neutropenia or liver or kidney toxicity. No patient received granulocyte colony-stimulating factor. The only infectious complication was a single urinary tract infection in a patient receiving 100 mg/m^2 MTX. Two patients (22.2%) developed lymphedema, one secondary to metastases to the arm.

Table 1. CHARACTERISTICS OF PATIENTS

Patient Number	MTX (mg/m^2)	Age at Dx (yr)	LN (clin/path)	ER/PR
1	40	72	No/10	−/−
2	40	40	No/0	+/+
3	60	44	Yes/0	−/−
4	60	39	No/10	−/−
5	60	52	No/1	−/+
6	80	60	Yes/8	+/−
7	80	51	Yes/0	+/−
8	100	41	No/1	+/−
9	100	47	No/0	+/+

MTX = methotrexate; Dx = diagnosis; LN = lymph nodes; clin = clinical; path = pathological; ER = estrogen receptor; PR = progesterone receptor.

Table 2. RECURRENCES, METASTASES, AND SURVIVAL

Patient Number	Recurrence	Metastases	Survival
1	No	Brain only	57
2	No F/U	—	—
3	No	No	48
4	No	Arm, pleura	25†
5	Chest wall*	No	29
6	No	No	53
7	No	No	41
8	No	Multiple	17†
9	No	Brain only	9†

* Radiation therapy not given as primary therapy.
† Breast cancer-related deaths.

Three patients had clinically positive axillary lymph nodes before starting MTX treatment. Axillary lymph node involvement was absent in four (44.4%) of the nine patients. Two of the 5 patients had a single axillary lymph node positive; 3 had 8 to 10 axillary lymph nodes positive. Two patients were ER+/PR+, four were ER+/PR−, one was ER−/PR+, and three were ER−/PR−. Patients received tamoxifen unless they were ER and PR receptor-negative. Eight of the nine patients responded to the chemotherapy regimen. Two patients had a complete clinicopathologic response with no evidence of tumor in the mastectomy specimen, three had a partial response (≥50%), three had minimal tumor response (20% to <50%) and one had no significant response.

None of the eight patients who received XRT had a recurrence on the chest wall. One patient not receiving XRT developed a chest recurrence at 1 year, was treated with XRT, and remains disease-free. Four patients developed metastases (9 months, 1 year, 2 years, and 3 years out). Two of these four patients developed brain metastases as the only site of metastasis, one in the 40 mg/m^2 and the other in the 100 mg/m^2 group. The patient on 40 mg/m^2 developed brain metastases 3 years after the diagnosis, was treated with XRT and surgery, and 1 year later (4 years after diagnosis) is still alive. The other two patients who developed metastases did so in multiple sites (bone, liver, brain, skin, pleural effusion), one in the 60 mg/m^2 and the other in the 100 mg/m^2 group. There were a total of three deaths (Table 2).

DISCUSSION

There is growing clinical evidence of the therapeutic value of high-dose chemotherapy regimens.[2–4] However, chemotherapeutic regimens are limited by the toxicity to the normal host tissues.[4] MTX is one of the most commonly used antineoplastic drugs and has been shown to cause structural and functional injury to the gastrointestinal tract, manifested clinically by severe enterocolitis. Fox et al.[9,10] showed that the morbidity and mortality of MTX administered to rats were ameliorated by the oral administration of GLN. Klimberg et al.[19] demonstrated in a rat sarcoma model that GLN does not stimulate tumor growth and in fact decreases growth in implantable as well as carcinogen-induced breast models.[20,21] Clinical application of these findings has been inhibited by concern that GLN would protect not only the host but also the tumor, thereby reducing the chemotherapeutic effectiveness of MTX. Klimberg et al.[11] demonstrated in a rat model that oral GLN supplementation enhances the tumoricidal action of MTX, in addition to reducing bacteremia and improving survival.

The mechanism by which GLN facilitates MTX effectiveness is its ability to increase MTX concentration in tumor cells.[13] The authors hypothesized that GLN increases the concentration of MTX in tumor cells by increasing the amount of polyglutamated MTX as well as the number of polyglutamations. Polyglutamation impairs the efflux of MTX from tumor cells. Considerable heterogeneity has been observed in the capacity of cultured human breast cancer to synthesize MTX polyglutamates, but in a majority of cell lines, significant conversion to polyglutamate derivatives occurs.[22,23] *In vitro* evidence exists that increases in GLN concentration cause proportional increases in the ratio of polyglutamated to monoglutamated MTX, thus improving retention of MTX within the tumor.[24]

In the current rat model, the authors found that in the GLN group, there was a threefold increase in total MTX in the tumor; this increase was a result of an absolute increase (nmol/g) in both the diglutamate and pentaglutamated MTX (see Figs. 3A and 4). Further, the pentaglutamated MTX was 14-fold higher in the tumor in the GLN group compared to the nonsupplemented group (see Fig. 4). Significantly, these changes were associated with no differences in the concentration of MTX in the serum between the GLN and the control group at this 24-hour time period.

If GLN increases the concentration of MTX in the tumor, is there a similar change in the gut that would be detrimental? This would be contrary to the previously cited studies, which have shown protective effects of GLN against MTX-induced enterocolitis.[9–12] In the present study, HPLC assays for polyglutamates MTX$^{1-7}$ showed a significant decrease in total mean polyglutamated MTX in the gut of GLN-fed animals compared to the control group (see Fig. 3A). Specifically, in the gut there was a significant decrease in MTX.[6] These data do not exclude a differential uptake of various forms of MTX and folate in the gut *versus* the tumor. The gut, known to prefer the monoglutamated form of folate, may prefer lesser glutamated forms of MTX.[25]

Another alternative is that GLN may protect the gut by regulating glutathione (GSH) synthesis. GSH has a protective effect against oxidant injury. Austgen et al.[26] have shown increases of GSH in the gut without commensurate increases in GSH levels in tumors with supplemental intravenous GLN in a sarcoma-bearing rat model. Rouse et al.[27] have shown that in animals treated with MTX and supple-

mental GLN, there was decreased intracellular tumor GSH and increased GSH in multiple host tissues when compared to controls. The findings of these animal experiments suggest that GLN may be of therapeutic as well as nutritional value.

Given the results of the preclinical animal studies, the authors performed a phase I trial to investigate the safety of oral GLN to allow dose escalation of MTX in inflammatory breast cancer patients. Studies in humans have demonstrated the safety of GLN-supplemented oral as well as total parenteral nutrition in normal volunteers.[28] Several trials have examined the effects of intravenous GLN on morbidity and mortality in bone marrow transplant patients as well as in patients with solid tumors.[29,30] Limited nonrandomized trials have been published using oral GLN for patients receiving chemotherapy,[31] and a small randomized trial showed benefit in patients receiving XRT.[32]

The authors performed this phase I trial to study the toxicities and problems associated with oral GLN supplementation with escalating doses of MTX in inflammatory breast cancer patients. No toxicity of oral GLN itself was detected in any patient. A number of toxicities have been described after the administration of high-dose MTX including myelosuppression, reversible nephrotoxicity, chemical hepatitis (manifest as elevation of transaminases in up to 60% of courses) and hyperbilirubinemia.[33] Mucositis and dermatologic toxicity, ranging from mild transient erythematous eruptions to frank exfoliative dermatitis, occurs in up to 30% of patients on 40 mg/m^2 MTX regimens.[34] Only one of the nine patients in this study developed a grade I mucositis (at 80 mg/m^2). There were no liver or renal toxicities, and none of the patients required granulocyte colony-stimulating factor for neutropenia.

Median follow-up of living patients was 47 months. Median survival of all patients was 29 months. The patient who died at 9 months probably had metastases at diagnosis; excluding this patient, the median survival was 35 months. This is a phase I trial and as such was not meant to be a survival study. However, the results compare favorably with those of other published series, with survival averaging 35 months.[35] Singletary et al.[36] analyzed the prognostic factors in patients with inflammatory breast cancer and concluded that the clinical and histologic response to chemotherapy is significantly related to overall survival. In the current study, two of the nine patients had a complete response and four of nine had negative lymph nodes. Interestingly, the two patients who had a complete clinicopathologic response developed brain metastases; one died from the cancer and the other remains disease-free after XRT and neurosurgery. In clinical studies, high-dose MTX has generally not been effective in preventing central nervous system relapse.[37]

Initial treatment as well as limited follow-up demonstrates that oral GLN supplementation is safe in its delivery to the tumor-bearing host. The authors' studies suggest that GLN produces both increased initial delivery of the drug to the tumor and decreased host toxicity from the chemotherapeutic treatment. Further studies are necessary to determine if GLN may also play an important role in the clinical responsiveness of cancer to these agents.

References

1. Souba WW, Wilmore DW. Diet and nutrition in the care of the patient with surgery, trauma, and sepsis. In: Shells ME, Young VR, eds. Modern Nutrition in Health and Disease. Philadelphia: Lea & Febiger; 1988:1306–1336.
2. Hryniuk WM. Average relative dose intensity and the impact on design of clinical trials. Semin Oncol 1987; 14:65–74.
3. Yap HY, Blumenschein GR, Yap BS, et al. High-dose methotrexate for advanced breast cancer. Cancer Treat Rep 1979; 63:757–761.
4. Nirenberg A, Mosende C, Mehta BM, et al. High-dose methotrexate with citrovorum factor rescue: predictive value of serum methotrexate concentrations and corrective measures to avert toxicity. Cancer Treat Rep 1977; 61:779–783.
5. Frisell WR. Synthesis and catabolism of nucleotides. In: Frisell WR, ed. Human Biochemistry. New York: MacMillan; 1982:292–304.
6. Newsholme EA, Crabtree B, Ardawi MSM. Glutamine metabolism in lymphocytes: its biochemical, physiological and clinical importance. Q J Exp Physiol 1985; 70:473–489.
7. Windmueller HG. Glutamine utilization by the small intestine. Adv Enzymol Relat Areas Mol Biol 1982; 53:202–231.
8. Zeilke RH, Ozand PT, Tildon JT, et al. Reciprocal regulation of glucose and glutamine utilization by cultured human diploid fibroblasts. J Cell Physiol 1985; 70:473–489.
9. Fox AD, Kripke SA, Berman JR, et al. Reduction of the severity of enterocolitis by glutamine-supplemented enteral diets. Surg Forum 1987; 38:43–45.
10. Fox AD, Kripke SA, DePaula J, et al. Effect on a glutamine-supplemented enteral diet on methotrexate-induced enterocolitis. JPEN 1988; 12(4):325.
11. Klimberg VS, Nwokedi E, Hutchins LF, et al. Does glutamine facilitate chemotherapy while reducing its toxicity? Surg Forum 1991; 42:16–18.
12. Klimberg VS, Nwokedi E, Hutchins LF, et al. Glutamine facilitates chemotherapy while reducing toxicity. JPEN 1992; 16(6):83S–87S.
13. Klimberg VS, Pappas AA, Nwokedi E, et al. Effect of supplemental dietary glutamine on methotrexate concentrations in tumors. Arch Surg 1992; 127:1317–1320.
14. Bergmeyer HU, ed. Methods of Enzymatic Analysis. New York: John Wiley & Sons; 1974:1704–1708.
15. Lowry OH, Rosenbrough WJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265–275.
16. Windmueller HG. Phosphate-dependent glutaminase in the small intestine. Arch Biochem Biophys. 1977; 182:506–517.
17. Varela-Moreiras G, Seyoum E, Selhub J. Combined affinity and ion pair liquid chromatographies for the analysis of folate distribution in tissues. J Nutr Biochem 1991; 2:44–53.
18. Jolivet J, Schilsky RL. High-pressure liquid chromatography analysis of methotrexate polyglutamates in cultured human breast cancer cell. Biochem Pharm 1981; 30(11):1387–1390.
19. Klimberg VS, Souba WW, Salloum RM, et al. Glutamine-enriched diets support muscle glutamine metabolism without stimulating tumor growth. J Surg Res 1990; 48(4):319–323.
20. Fahr M, Kornbluth J, Blossom S, et al. Glutamine enhances immunoregulation of tumor growth. JPEN 1994; 18(6):471.
21. Feng Z, Cao Y, Kornbluth J, et al. Glutamine prevents DMBA-induced breast cancer growth. Surg Forum 1996; 47:524–526.

22. Jolivet J, Schilsky RL, Bailey BD. The synthesis and retention of methotrexate polyglutamates in cultured human breast cancer cells. Ann NY Acad Sci 1982; 397:184–192.
23. Ackland SP, Schilsky RL. High-dose methotrexate: a critical reappraisal. J Clin Oncol 1987; 5(12):2017–2031.
24. Fry DW, Gewirtz DA, Yalowich JC, Goldman ID. Characteristics of the accumulation of methotrexate polyglutamate derivatives in Ehrlich ascites tumor cells and isolated rat hepatocytes. Adv Exp Med Biol 1983; 163:215–234.
25. Goldman ID. The membrane transport of methotrexate (NSC-740) and other folate compounds: relevance to rescue protocols. Cancer Chemother Rep (Part 3) 1975; 6(1):63–72.
26. Austgen TR, Dudrick PS, Sitren H, et al. The effects of glutamine-enriched total parenteral nutrition in tumor growth and host tissues. Ann Surg 1992; 215:107–113.
27. Rouse K, Nwokedi E, Woodliff J, et al. Glutamine enhances selectivity of chemotherapy through changes in glutathione metabolism. Ann Surg. 1995; 221(4):420–426.
28. Ziegler TR, Benfell K, Smith RJ, et al. Safety and metabolic effects of L-glutamine administration in humans. JPEN 1990; 14(suppl):137S–146S.
29. Schloerb PR, Amare M. Total parenteral nutrition with glutamine in bone marrow transplantation and other clinical applications (a randomized, double-blind study). JPEN 1993; 17(5):407–413.
30. Ziegler TR, Young LS, Benfell K, et al. Glutamine-supplemented parenteral nutrition improves nitrogen retention and reduces hospital mortality *versus* standard parenteral nutrition following bone marrow transplantation: a randomized, double-blind trial. Ann Intern Med 1992; 116:821–828.
31. Skubitz KM, Anderson PM. Oral glutamine to prevent chemotherapy-induced stomatitis: a pilot study. J Lab Clin Med 1996; 127(2):223–228.
32. Richards EW, Long CL, Pinkston JA, et al. The role of oral glutamine supplementation in the prevention of radiation-induced enterocolitis in prostate cancer patients. FASEB J 1992; 6(5):A1680.
33. Ihde DC, Johnston-Early A, Carney DN. Lack of efficacy of high-dose methotrexate by 30-hour infusion in patients with progressive small cell carcinoma of the lung. Cancer Treat Rep 1982; 66:1223–1225.
34. Doyle LA, Berg C, Bottino G, et al. Erythema and desquamation after high-dose chemotherapy. Ann Intern Med 1983; 98:611–613.
35. Lopez MJ, Porter KA. Inflammatory breast cancer. Surg Clin North Am 1996; 76(2):411–429.
36. Singletary SE, Ames FC, Buzdar AU. Management of inflammatory breast cancer. World J Surg 1994; 18:87–92.
37. Hande KR, Oldham RK, Fer MF, et al. Randomized study of high-dose chemotherapy in the treatment of extensive small cell lung cancer. Am J Med 1982; 73:413–419.

Discussion

DR. EDWARD M. COPELAND, III (Gainesville, Florida): Thank you, Dr. Wells. If you will allow me to brag a little bit, Dr. Klimberg's work on the effect of glutamine ingestion on tumor dynamics represents a further extension of her studies done while in our laboratories at the University of Florida when she was a surgical house officer. We are very proud of her.

In 1972, Dr. Meyer demonstrated that methotrexate had greater cell penetrance in an acid environment that he created by inducing lactic acidosis. Earlier, in 1924, Warberg and colleagues showed that *in vitro* glycolysis was progressively inhibited by a falling pH and ceased to function at a pH below 6. In my early days of clinical investigation with total parenteral nutrition (TPN), Stan Dudrick and I postulated that a vicious cycle might occur within a cancer cell forced to metabolize large concentrations of glucose. As more glucose is metabolized, more lactic acid is generated which might cause a fall in interstitial and cellular pH; the tricarboxylic acid cycle then ceases to function and more dependence on anaerobic glycolysis would ensue, thus producing more lactic acid and a further fall in pH. Over the years, TPN has been shown to improve nutrition in patients with cancer but is not an adjunct to chemotherapy. In other words, giving patients with cancer large volumes of glucose via TPN has not improved response to chemotherapeutic agents when compared to a well nourished control group. Until now, TPN has not contained glutamine.

Dr. Klimberg and her colleagues postulate that glutamine increases methotrexate tumorocidal effect by concentrating methotrexate intracellularly in its glutamated form. I would ask her to comment on the possible change in intracellular pH in a cancer cell exposed to higher concentrations of intracellular glutamine and its byproducts.

Several investigators have demonstrated that glutamine ingestion does not affect tumor growth in a rodent model, but the aneuploid/diploid ratio has been shown to increase under glutamine stimulation. Did you investigate ploidy in your studies?

The lack of toxicity from methotrexate in the patient study is most impressive. The response rates and survival are similar to those reported in other published series. Obviously, a randomized prospective trial will be necessary to determine the effect of glutamine in patients receiving methotrexate. Since toxicity was reduced, differences could probably be identified with a relatively small sample of patients.

Last of all, tumor tissue can be obtained in patients with inflammatory breast cancer by core-needle biopsy. Did you determine the levels of methotrexate in specimens from the breast cancer patients?

Congratulations on a very nice study. I appreciate the opportunity to read it, Suzanne. [Applause]

DR. KIRBY I. BLAND (Providence, Rhode Island): Dr. Wells, Dr. Copeland, Members, and Guests of the Association. I, too, would like to add my congratulations to Dr. Klimberg, Dr. Westbrook, and their colleagues from Little Rock for bringing to the attention of the Association this important study for the application of nutrients to tumor-bearing hosts that are receiving high-dose chemotherapy to limit their toxicities at multiple sites. This is an important paper, and I commend them again for applying translational research to now put this into a phase one study that actually evaluates glutamine substrate to increase pharmaceutical concentration in tumor cells by enhancement of polyglutamated methotrexate.

Clearly, the extension of this bench research to humans signals immense progress in our care of patients with advanced neoplasms.

Suzanne, I have a number of questions for you. First, the absolute increase in nanomoles per gram of the methotrexate was evident in both diglutamated and pentaglutamated subsets. Also, if I read your paper correctly, it was 14-fold that of the intracellular tumor methotrexate for the pentaglutamated variant treated with glutamine when compared to your control which was glycine. So my question is, could you tell us at either a cellular or a molecular mechanism why you have such rapid transport or accelerated methotrexate transport after the administration of oral glutamine *versus* glycine?

Secondly, you have observed a significant decrease in the poly-